Peripheral amplification of sweating – a role for calcitonin gene-related peptide

Tanja Schlereth*, Jan Oliver Dittmar, Bianca Seewald and Frank Birklein

Department of Neurology, Johannes Gutenberg-University Langenbeckstr. 1, D-55101 Mainz, Germany

Neuropeptides are the mediators of neurogenic inflammation. Some pain disorders, e.g. complex regional pain syndromes, are characterized by increased neurogenic inflammation and by exaggerated sudomotor function. The aim of this study was to explore whether neuropeptides have a peripheral effect on human sweating. We investigated the effects of different concentrations of calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP) and substance P (SP) on acetylcholine-induced axon reflex sweating in healthy subjects (total $n = 18$). All substances were applied via dermal microdialysis. The experiments were done in a parallel setting: ACh alone and ACh combined with CGRP, VIP or SP in various concentrations were applied. Acetylcholine (10^{-2} M) always elicited a sweating response, neuropeptides alone did not. However, CGRP significantly enhanced ACh-induced sweating ($P < 0.01$). *Post hoc* tests revealed that CGRP in physiological concentrations of 10^{-7}–10^{-9} M was most effective. VIP at any concentration had no significant effect on axon reflex sweating. The duration of the sweating response ($P < 0.01$), but not the amount of sweat, was reduced by SP. ACh-induced skin blood flow was significantly increased by CGRP ($P < 0.01$), but unaltered by VIP and SP. The results indicate that CGRP amplifies axon reflex sweating in human skin.

(Resubmitted 28 June 2006; accepted after revision 17 August 2006; first published online 24 August 2006)
Corresponding author T. Schlereth, Department of Neurology, Johannes Gutenberg-University, Langenbeckstr. 1, D-55101 Mainz, Germany. Email: schleret@uni-mainz.de

Calcitonin gene-related peptide (CGRP) is a neuropeptide, which is produced by alternative splicing of the calcitonin gene, e.g. in primary afferent neurons. CGRP is involved in the generation of pain and hyperalgesia. In the spinal cord it contributes to nociceptive sensitization (Ma & Bisby, 1998; Bennett *et al.* 2000), in the periphery CGRP is a very potent vasodilator. If released from primary afferent fibres into the peripheral tissue, CGRP contributes to 'neurogenic inflammation', in particular to the flare response (Weidner *et al.* 2000). The flare is the area of vasodilatation which occurs in the surrounding of a skin injury when peripheral C-fibres are excited. Experimentally, a flare can be induced, for example, by electrical current or capsaicin injection (LaMotte *et al.* 1992; Schmelz & Petersen, 2001). CGRP induces arteriolar vasodilatation by binding mainly to CGRP 1 receptors on smooth muscle cells (Casey *et al.* 1997). The second messenger for CGRP-induced vasodilatation is nitric oxide (NO).

Some neuropathic pain states, in particular complex regional pain syndromes (CRPS) are clinically characterized not only by exaggerated neurogenic inflammation (Weber *et al.* 2001), but also by disturbances of the sympathetic nervous system, e.g. localized profuse sweating at the painful limb (Birklein *et al.* 1998). In these 'inflammatory' stages of CRPS, sweating in particular was increased if it was induced peripherally by axon reflex mechanisms (Birklein *et al.* 1997). Currently, there is no good functional explanation for this clinical phenomenon. Histological data using immunohistochemical staining revealed that CGRP is co-localized to acetylcholine in peripheral sudomotor axons, which innervate human sweat glands (Landis & Fredieu, 1986; Kruger *et al.* 1989). Furthermore, CGRP can also be found in the sweat glands themselves (Ishida-Yamamoto & Tohyama, 1990; Zancanaro *et al.* 1999). All these morphological findings suggest that CGRP might be involved in human sweating; however, well controlled functional data are missing.

Vasoactive intestinal peptide (VIP) is a peptide that acts predominantly as a neurotransmitter in the gastrointestinal system. VIP is also synthesized in neurons. In the central nervous system (CNS) it is important to coordinate daily rhythms in behaviour and physiology (Aton *et al.* 2005). In the peripheral tissue VIP acts as a vasodilator (Lundberg *et al.* 1981; Henning & Sawmiller, 2001). Compared with CGRP, however, VIP vasodilatation is less potent and shorter lasting (Brain *et al.* 1986). VIP was also found in neurons associated with human sweat glands (Hartschuh *et al.* 1984; Vaalasti *et al.* 1985). The functional role of VIP in human sweating is controversial. In rats,

 DOI: 10.1113/jphysiol.2006.116111

VIP itself induces sweating (Stevens & Landis, 1987) but in humans it does not (Yamashita *et al.* 1987; Berg *et al.* 1995).

Substance P (SP) is a tachykinin which is widely distributed in the peripheral and central nervous system (O'Connor *et al.* 2004). In the CNS, SP is involved in stress–anxiety responses of the amygdala (Ebner *et al.* 2004). In peripheral neurogenic inflammation it mainly causes the second component – plasma extravasation and oedema (Lembeck *et al.* 1982; Weidner *et al.* 2000; Sauerstein *et al.* 2000). Sympathetic ganglions and neurons innervating sweat glands also contain substance P – besides CGRP and VIP (Lindh *et al.* 1989*a*,*b*). In contrast to CGRP, preliminary human studies suggest that SP should have an inhibitory effect on sweating (Kumazawa *et al.* 1994; Berg *et al.* 1995).

The aim of the present investigation was therefore to elucidate the functional effects of neuropeptides (CGRP, VIP, SP) on human sweating. In order to avoid simultaneous skin trauma, which might affect neuropeptide content of the skin by C-fibre activation, we used a dermal microdialysis system. Microdialysis membranes are inserted into the skin. After subsiding of insertion-related skin changes, substances then can be atraumatically applied to the tissue via these microdialysis fibres. In previous investigations it has been shown that acetylcholine (ACh) application via dermal microdialysis is suitable to induce axon reflex sweating (Shibasaki & Crandall, 2001; Bickel *et al.* 2004). In addition to ACh, different concentrations of CGRP, VIP or SP were added. Thus, we were able to investigate the role of these neuropetides in the modulation of human sweating.

Methods

Written informed consent was obtained from all subjects according to the Declaration of Helsinki and the study was approved by the local ethics committee. Three series of microdialysis experiments were done. In the first series ($n = 39$ experiments) the effect of CGRP on sweating was investigated. CGRP was applied either alone ($n = 4$), in combination with ACh (10^{-2} M), or ACh (10^{-2} M) alone for control ($n = 34$ experiments). In the second series of experiments VIP ($n = 19$ experiments) and in the third series substance P (SP; $n = 18$ experiments) were investigated in the same way. We started with using CGRP. Due to pilot dose-finding experiments (not mentioned here) eight subjects (age, 25 ± 1 years; 7 females, 6 males) repeatedly participated in order to yield $n = 6$–8 for paired comparisons of each CGRP concentration + ACh with ACh alone. Six subjects repeatedly participated in experiments using VIP (age, 25.3 ± 3 years; 3 female, 3 male) and four subjects repeatedly participated in experiments using SP (age, 25.8 ± 2 years; 1 female, 3 male) in order to yield $n = 4$ for paired comparison of each VIP and SP concentration. Paired comparisons were chosen since sweating could be markedly variable between subjects (Schlereth *et al.* 2005*c*). Since serum neuropeptide concentrations did not change significantly during menstrual cycle (Goodnough *et al.* 1979; Ottesen *et al.* 1982; van Leeuwen *et al.* 1992; Kerdelhue *et al.* 1997) we did not control women for their menstrual cycle.

All subjects were placed comfortably in a chair. The left lower leg was fixed by a vacuum cushion. After cooling the skin, four microdialysis membranes were inserted pain-free into the skin of the medial aspects of the lower leg. The membranes were arranged in two groups of two membranes, the distance between the two membranes in each group was about 2 cm, and between the two groups about 5 cm. The microdialysis membranes, 200 μm in width, had a cut-off of 3000 kDa and the membranes were placed intradermally at a length of 15 mm (Schmelz *et al.* 1997). With this technique all substances could be applied without time-related skin trauma. Between the two membranes of each group, sweat chambers were fixed to the skin (see Fig. 1). Sweat chambers covered 3.125 cm^2 (1.25 cm × 2.5 cm) of skin. Dry nitrogen gas was passed through the chambers at a constant flow (90 ml min^{-1}). The relative humidity was measured downstream by capacitance hygrometers (Hygroscope DV 2, Rotronic AG, Basel, Switzerland).

During the baseline condition in the first hour after insertion, membranes were perfused with physiological saline at a constant flow of 4 μl min^{-1}. During the 40 min stimulation period in the second hour, two membranes (first group) were perfused with ACh (10^{-2} M) in saline, while the other two membranes (second group) were perfused with ACh (10^{-2} M) in combination with different concentrations of CGRP, VIP or SP dissolved in saline. The concentrations of CGRP varied from 10^{-6} to 10^{-10} M, the concentrations of VIP from 10^{-5} to 10^{-8} M and the concentrations of substance P from 10^{-6} to 10^{-9} M. After 40 min of stimulation, membranes were again perfused with saline for a 20 min washout period. The volume of stimulus-evoked sweating was quantified by calculating (1) the absolute humidity during the baseline period directly before stimulation and at the peak of the sweat reaction (Birklein *et al.* 1997) and (2) by measuring the area under the sweat curve from onset until the end of the sweating reaction (Birklein *et al.* 1997). The duration of the sweating response was measured in seconds.

All applied substances (ACh, CGRP, VIP and SP) caused vasodilatation to a variable degree. For measuring superficial skin blood flow laser Doppler imaging (LDI; Moor Instruments Limited, London, UK) was used. Perfusion pictures of both groups of membranes were taken every 5 min during the stimulation period. The scanner distance to the skin was 50 cm. Laser signals were

stored in a PC for further computation using dedicated software (moorLDI 3.01; Moor Instruments). Offline, mean flux was calculated in an approximately 10 cm^2 area of skin including membranes and the surrounding skin, which was not covered by the sweat chambers.

Control experiments to estimate diffusion capacity of ACh in human skin

In previous experiments we have been able to exclude that ACh, which is released from sudomotor fibres, reaches the dermal microdialysis membranes. All ACh, which occurred in the eluate, is of non-neuronal origin, e.g. from keratinocytes (Schlereth *et al.* 2006). In order to assess the diffusion capacity of ACh in the skin we therefore performed experiments in which we quantified ACh in the eluate from membranes 2 and 30 cm away from membranes, which were perfused with 10^{-2} M ACh, alone ($n = 4$) and in combination with the cholinesterase inhibitor neostigmine (10^{-5} M) ($n = 3$).

Statistical analysis

All values are given as mean and standard error (S.E.M.). For statistical analysis a repeated measures ANOVA was performed in order to assess the neuropeptide effects. Since sphericity could not be assumed we used Greenhouse-Geisser correction. In order to allocate significant neuropeptide concentrations, *t* tests for paired samples were used as *post hoc* tests. Statistical analysis was performed with the SPSS software package (release 12.0, SPSS Inc., Chicago, IL, USA). Significance was considered at $P < 0.05$.

Results

CGRP ($n = 39$ experiments)

These experiments were performed in eight subjects. Acetylcholine (10^{-2} M) alone always induced sweating. In contrast, CGRP alone at concentrations of 10^{-5}–10^{-7} M did not induce sweating in our experiments ($n = 4$, data not shown). However, CGRP amplified ACh-induced sweating. The sweat response, measured as absolute humidity at the peak ($F = 10.4$, $P < 0.005$), the area under the sweat curve ($F = 8.4$, $P < 0.01$) and the duration of the sweat response ($F = 10.4$, $P < 0.005$) was increased when CGRP was added to ACh (see Figs 1 and 2). *Post hoc* analysis revealed that CGRP particularly enhanced sweating in physiologically reasonable concentrations from 10^{-7} to 10^{-9} M (for details see Fig. 2*A*). In order to rule out the possibility that the differences between CGRP and ACh stimulation depend on different results of ACh stimulation in our paired comparison setting rather than on a true CGRP effect, we additionally analysed sweating after ACh stimulation alone. There was no significant effect of the different sessions on ACh-induced sweating ($F < 1.6$ for all sweat measures, all not significant (n.s.)).

Skin blood flow measured as mean flux in the skin area of interest was compared between stimulation with CGRP + ACh and ACh alone. CGRP significantly increased skin perfusion ($F = 31.5$, $P < 0.001$). *Post hoc* tests revealed that in particular 10^{-7} M ($F = 3.6$, $P < 0.01$) and 10^{-9} M CGRP ($F = 3.7$, $P < 0.01$) led to significantly increased skin blood flow (see Fig. 3*A*). In a similar way to the sweating results, we also assessed the possibility that ACh stimulation alone could be responsible for the significant differences in our paired setting rather than the CGRP effect. There was again no significant difference of

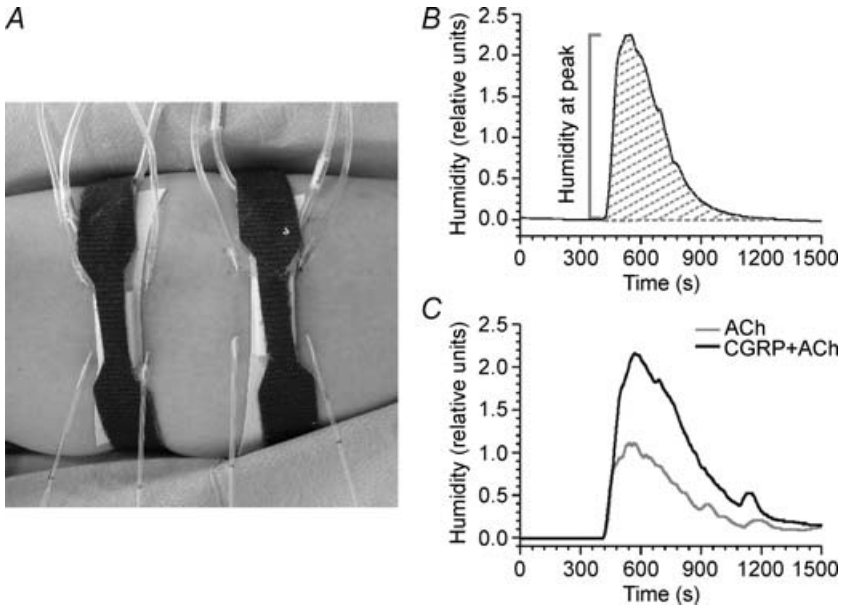

Figure 1. Measurement of sweating
A, two sweat chambers were attached to the skin. Each chamber was surrounded by two microdialysis membranes. The sweat chambers were perfused with dry nitrogen gas. Humidity was measured with hygrometers and assessed as sweat rate over time. *B*, example of a sweat curve of one subject. The area under the sweat curve (area hatched in grey) and the absolute humidity measured at the peak of the sweating response (marked by the grey line) were evaluated individually. *C*, example of sweat curves of one subject. CGRP at a concentration of 10^{-9} M in combination with ACh induced a stronger sweating response than ACh alone.

ACh-induced vasodilatation between the different sessions ($F = 0.3$, n.s.).

VIP ($n = 19$ experiments)

The whole series of VIP experiments was performed in six subjects. VIP alone (10^{-5} and 10^{-6} M, $n = 3$) did not induce sweating (data not shown). VIP was added to ACh (10^{-2} M) in concentrations of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M and sweating was compared with ACh alone ($n = 4$ for each concentration). Not absolute humidity at the peak of the sweat curve ($F = 0.5$, n.s.), nor the area under the curve ($F = 1.5$, n.s.) nor the duration of the sweat response ($F = 1.2$, n.s.) were different between ACh and the combination of VIP and ACh (see Fig. 2*B*). There was again no significant effect of the different sessions on ACh-induced sweating ($F < 0.9$ for all sweat measures, all n.s).

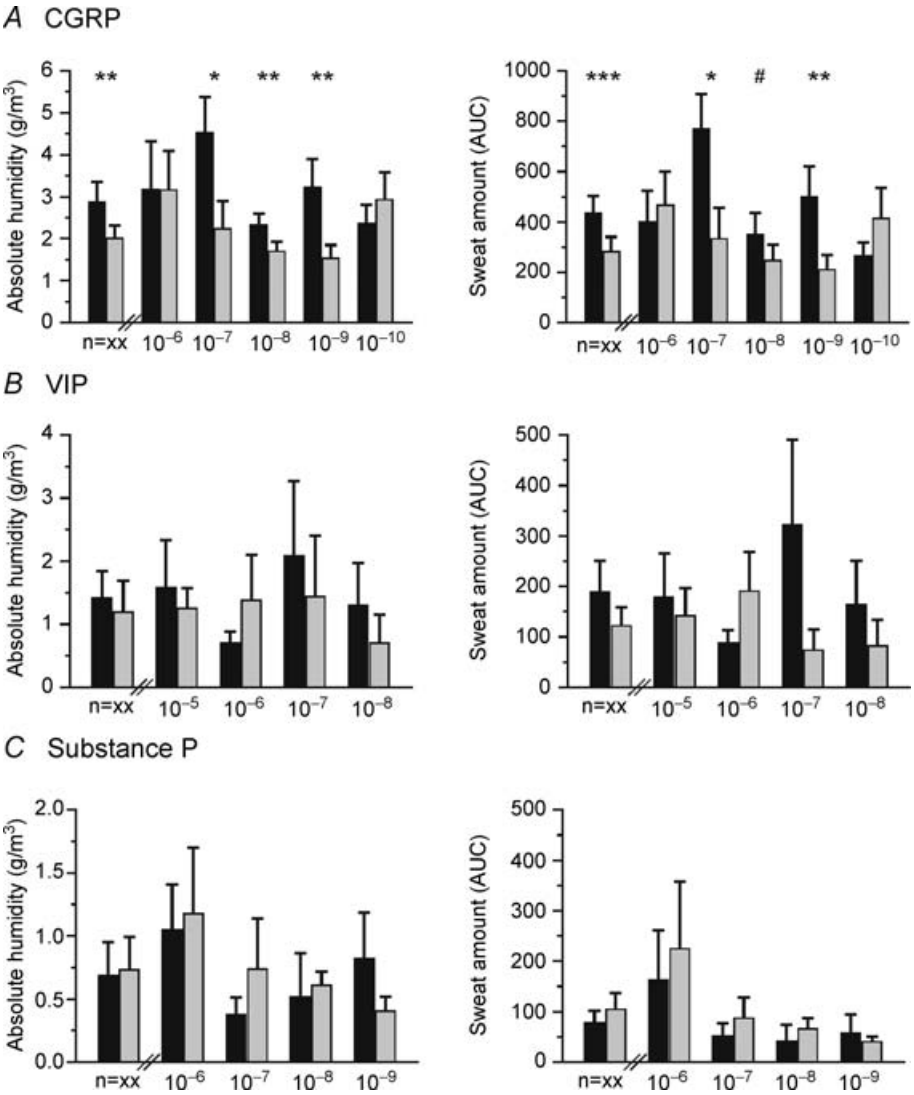

Figure 2. Evaluation of the sweating reaction
Sweating responses of all subjects. Area under the curve (AUC) and absolute humidity at peak were evaluated. $n = $ xx: mean of all concentrations. *A*, CGRP at concentrations of 10^{-6}–10^{-10} M in combination with ACh (black bars) compared with 10^{-2} M ACh alone (grey bars). At concentrations of 10^{-6} and 10^{-10} M sweating is reduced with CGRP, but at concentrations of 10^{-7}–10^{-9} M sweating is enhanced significantly with CGRP. Taken all concentrations together sweating is enhanced significantly with CGRP also. *B*, VIP at concentrations of 10^{-5}–10^{-8} M in combination with ACh (black bars) compared with 10^{-2} M ACh alone (grey bars). No significant difference in sweat rate between VIP and ACh could be detected. *C*, substance P at concentrations of 10^{-6}–10^{-9} M in combination with ACh (black bars) compared with 10^{-2} M ACh alone (grey bars). No significant difference in sweat rate between SP and ACh could be detected. *Post hoc t* test: $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, $\#P < 0.09$.

In accordance with the sweating results, VIP in addition to ACh did not significantly change skin blood flow ($F = 0.1$, n.s.; see Fig. 3*B*). There was again no difference in ACh-induced vasodilatation between the different sessions ($F = 0.9$, n.s.)

SP ($n = 18$ experiments)

These experiments were performed in four subjects. Substance P alone (10^{-5} and 10^{-6} M, $n = 2$) did not induce sweating (data not shown). SP was added to ACh (10^{-2} M) in concentrations of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} M and sweating was compared with ACh alone ($n = 4$ for each concentration). Neither absolute humidity at the peak of the sweat curve ($F = 0.07$, n.s.) nor the area under the curve ($F = 1.8$, n.s.) were different between ACh and the combination of SP and ACh (see Fig. 2*C*), but the duration of the sweat response was significantly shorter when SP and ACh were given in combination ($F = 9.5$, $P < 0.01$). The differences were small that *post hoc* tests were not able to detect them at single SP concentrations. Similar to the other experiments, there was again no significant effect of the different sessions on ACh-induced sweating ($F < 1.9$ for all sweat measures, all n.s.).

SP in addition to ACh did not significantly change skin blood flow ($F = 0.6$, n.s.; Fig. 3*C*). There was again no difference in ACh-induced vasodilatation between the different sessions ($F = 0.2$, n.s.).

Diffusion of ACh in human skin

At baseline, acetylcholine concentration ($n = 4$) was 1.7 pmol μl^{-1} in the microdialysis eluate of the two membranes 2 cm apart from the stimulation membrane. After perfusing the stimulation membrane with 10^{-2} M ACh, ACh concentration increased to a mean of 3.6 pmol μl^{-1}. ACh in the eluates from membranes 30 cm away did not increase (0.5 pmol μl^{-1}). When neostigmine (10^{-5} M) was added in order to block cholinesterases and to increase ACh recovery, results were the same: basal ACh increased from a mean of 103 pmol μl^{-1} to 451 pmol μl^{-1} in the nearby membranes.

Discussion

The results presented herein suggest that CGRP, which is usually released from primary afferent neurons in human skin, amplifies sweating. Hyperhidrosis occurs at CGRP concentrations of 10^{-7}–10^{-9} M, which have been shown to induce local vasodilatation (Weidner *et al.* 2000). Based on microdialysis extraction studies (Sauerstein *et al.* 2000; Kramer *et al.* 2005), it is also possible to calculate that about 10^{-8} M of CGRP is dissolved in a volume of 1 mm^3 of human skin *in vivo*. This further emphasizes

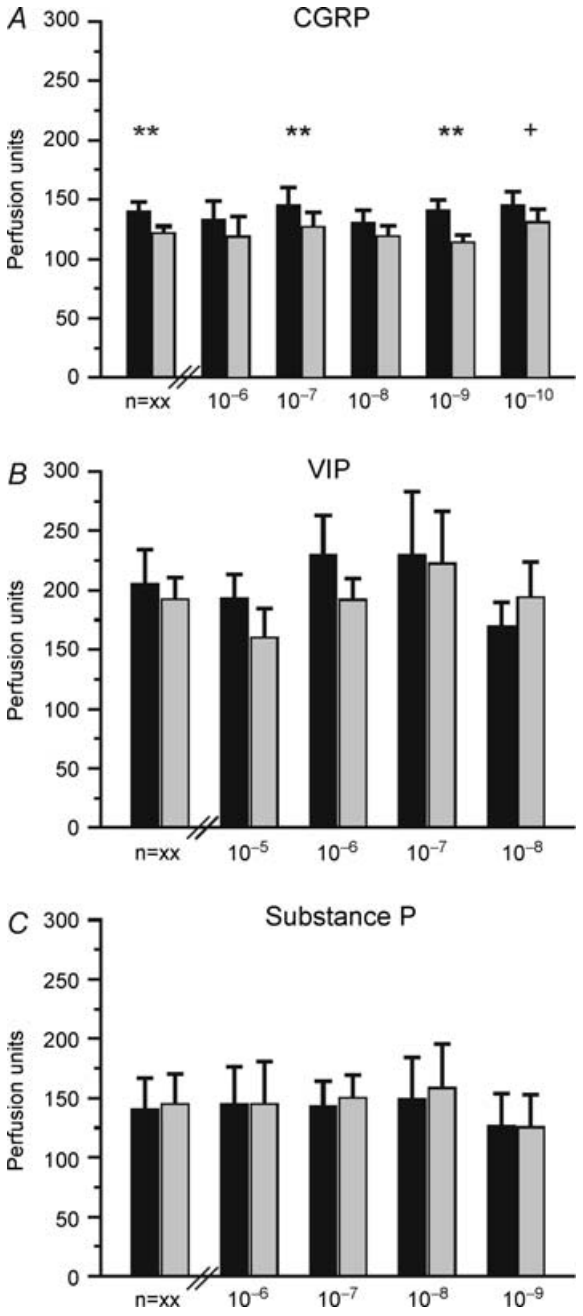

Figure 3. Superficial skin blood flow measured with laser-Doppler imaging
Mean skin blood flow for the time of the stimulation period (40 min). Mean ± S.E.M. of all subjects. Skin blood flow was measured in perfusion units. n = xx: mean of all concentrations. *A*, CGRP at concentrations of 10^{-6}–10^{-10} M (black bars) was tested against 10^{-2} M ACh alone (grey bars). CGRP enhanced skin blood flow at all concentrations, but this enhancement was only significant for 10^{-7} and 10^{-9} M and if all concentrations were taken together. *B*, VIP at concentrations of 10^{-5}–10^{-8} M (black bars) was tested against 10^{-2} M ACh alone (grey bars). No difference was significant. Only at the highest VIP concentrations (10^{-5} and 10^{-6} M) could a trend for enhanced skin perfusion with VIP be found. *C*, substance P at concentrations of 10^{-6}–10^{-9} M (black bars) was tested against 10^{-2} M ACh alone (grey bars). No significant differences in superficial skin blood flow could be found. *Post hoc t* test: $^{**}P < 0.01$, $^{+}P < 0.06$.

the physiological relevance of our findings, e.g. for pain disorders, which are characterized by enhanced CGRP release, vasodilatation and hyperhidrosis (Birklein *et al.* 2001). CGRP amplification of sweating, however, requires at least some concomitant sudomotor activation since CGRP alone was insufficient to induce sweating. VIP, another neuropeptide of the peripheral nervous system, did not alter ACh-induced sweating, and SP even showed modest inhibitory effects. Since our experimental setting excludes systemic effects, our findings provide evidence for a peripheral mechanism of modification of sweating in human skin (Schlereth *et al.* 2005*a*).

Effects on sweating

Human skin is unique in respect to sweating. In contrast to most animals, human hairy skin is important for active heat dissipation (Ogawa & Low, 1997). Thus, any comparison of thermoregulatory sweat glands in human hairy skin to sweat glands in hairy animals, which are usually restricted to foot pads in order to maintain skin elasticity, must be performed with caution. There is immunohistochemical evidence from human skin that; (1) CGRP positive nerve endings are located in the vicinity of sweat glands (Kennedy *et al.* 1994; Schulze *et al.* 1997), (2) CGRP is co-localized to ACh in peripheral sudomotor nerve fibres (Lindh & Hokfelt, 1990) and (3) CGRP occurs in human sweat glands (Zancanaro *et al.* 1999). In addition, recent data suggest that CGRP receptors are located on myoepithelial cells of human sweat glands (Hagner *et al.* 2002). These anatomical data already suggest that CGRP might be involved in peripheral regulation of sweating in humans. The functional results presented herein, now confirm this assumption – ACh-induced axon reflex sweating was increased in the presence of CGRP. Previous studies, however, revealed conflicting results. In accordance with the present findings, the application of CGRP alone did not induce sweating, but the combination with methacholine, an agonist for post-junctional muscarinic ACh receptors, which are located on sweat glands, induced a stronger sweat response (Kumazawa *et al.* 1994, 1997). In these studies, methacholine was injected alone and in combination with CGRP, and sweating was measured quantitatively with hygrometry – according to our study design. Just the opposite, i.e. a reduction of the nicotine-induced axon reflex sweating by CGRP, but also by VIP, was found by another group (Tanaka *et al.* 1990). In this investigation, however, the number of sweat droplets marked by iodine starch staining after injection of nicotine with or without CGRP was counted while sweating was not quantitatively assessed. There might be several explanations for these diverging results. The most likely one is that CGRP indeed enhances the sweat production in single sweat glands (Sato, 1997), which could be measured by quantitative hygrometry. On the other hand, CGRP has no effect on the number of activated sudomotor fibres, the number of activated sweat glands, or the anatomical structure of peripheral sudomotor innervation (Schlereth *et al.* 2005*b*). This is not surprising since even on primary afferent neurons CGRP has no powerful exciting effect (Weidner *et al.* 2000). Alternatively, CGRP could differently affect axon reflex sweating (by nicotine) and the direct sweat response (by methacholine). CGRP has been shown to inhibit nicotinic ACh receptors in rat cell preparations (Giniatullin *et al.* 1999; Nistri & Di Angelantonio, 2002; Di Angelantonio *et al.* 2003). Smaller fragments of CGRP ($CGRP_{1–6}$, $CGRP_{1–5}$ or $CGRP_{1–4}$), however, which occur after degradation of CGRP by peptidases in human tissue, enhance the activity of nicotinic ACh receptors. Thus, there might be very differential effects, depending on the predominant form of CGRP in the tissue. This could explain why high concentrations of CGRP have no hyperhidrotic effects in our study. The peptidase activity is not sufficient to degrade CGRP rapidly and inhibitory effects of the whole molecule and facilitory effects of the degredation products could neutralize themselves.

ACh also has direct muscarinergic properties on sweat glands, and in our study ACh was applied in pharmacological concentrations. Even though ACh undergoes rapid degrading by cholinesterases, we surprisingly were able to prove some diffusion of ACh over 2 cm, even if other substances like histamine do not seem to diffuse over longer distances (Petersen *et al.* 1997; Clough *et al.* 2002; Boutsiouki *et al.* 2004). Apart from diffusion the measured ACh could have been non-neuronal ACh released from skin cells like keratinocytes (. Schlereth *et al.* 2006). This means that muscarinic direct stimulation of sweat glands in the vicinity of the ACh application could at least also be possible, although previous studies have not shown functional relevance of such a diffusion in untreated skin (Low *et al.* 1983). CGRP receptors are expressed on myoepithelial cells of human sweat glands (Hagner *et al.* 2002). Therefore, an enhancement of sweat output due to increased squeeze out of sweat glands might be possible. Even if CGRP can be found in sudomotor fibres (Landis & Fredieu, 1986; Tainio *et al.* 1987; Dalsgaard *et al.* 1989), no direct evidence for functional CGRP receptors on sudomotor fibres has so far been found. We have been able to confirm this, since CGRP alone was unable to induce sweating. Therefore ACh and CGRP must act synergistically (Lindh & Hokfelt, 1990).

Another significant difference between the results of Tanaka *et al.* (1990) and ours might be the route of application of substances. We used dermal microdialysis, which allows the gradual application without time-related skin trauma (Sauerstein *et al.* 2000; Zahn *et al.* 2003). Tanaka *et al.* injected CGRP and nicotine at the same time. In particular, nicotine injection is painful, it excites

C-fibres and CGRP is quickly released (Schmelz *et al.* 1997). Thus, skin concentration of CGRP might be higher than assumed. Besides the inhibitory effects of high concentrations of CGRP in human skin, as discussed above, high CGRP concentration further induces vasodilatation also in deeper skin layers and the injected nicotine could be cleared before it reaches sudomotor fibres. This effect could also contribute to the finding that in our study highest concentrations of CGRP were less effective. Another unavoidable problem with injection is the fluid, which causes extracellular oedema, obstructs sweat ducts and might thereby hamper sweat production. The functional relevance of such a mechanism is clinically shown by tap water iontophoreses, the standard treatment of palmoplantar hyperhidrosis (Togel *et al.* 2002). Extracellular oedema is completely prevented by dermal microdialyis, since application of substances solely depends on osmotic diffusion.

Similar to the findings of CGRP, histological studies revealed that VIP is co-localized with ACh in human sudomotor fibres (Lundberg *et al.* 1980; Vaalasti *et al.* 1985; Heinz-Erian *et al.* 1986) and sweat glands (Kummer *et al.* 1990). These anatomical data suggest a functional role of VIP for the control of sweating. However, no clear evidence for this assumption has been provided so far. Although the injection of VIP induced sweating in rats, which could be blocked by atropine (Stevens & Landis, 1987) and VIP enhances the *ex vivo* sweat rate of single sweat glands from monkeys (Sato & Sato, 1987), the results in humans were ambiguous. Increased methacholine- induced sweating was observed using quantitative sudometry (Yamashita *et al.* 1987), but also reduced ACh-induced sweating by counting sweat spots (Tanaka *et al.* 1990; Berg *et al.* 1995). In neither study was VIP alone sufficient to evoke a sweating response in humans. Our results suggest that VIP has no significant effect on axon reflex sweating – neither enhancing nor reducing. Since ACh iontophoresis is a very strong stimulus for sweating, our results of course could not exclude that VIP participates in the regulation of physiological sudomotor activity. However, in skin biopsies from patients with familial dysautonomia, which is clinically characterized by impaired sweating, only CGRP immunoreactivity was reduced while VIP immunoreactivity was unchanged (Hilz *et al.* 2004). This would confirm our results that CGRP has an influence on human sweating while VIP has not.

Substance P is a tachykinin, which mainly acts on postcapillary venules via NK1 receptors and induces plasma leakage (oedema) (Leis *et al.* 2003). SP can be also found in sweat glands (O'Flynn *et al.* 1989; Zancanaro *et al.* 1999) as well as in sympathetic ganglions and sympathetic neurons innervating sweat glands (Lindh *et al.* 1989*a*,*b*). In general, the distribution of SP immunoreactivity around sweat glands is sparser than for CGRP or VIP (Lindberger *et al.* 1989; Kennedy *et al.* 1994) and in some studies no immunoreactivity for SP was found at all (Tainio *et al.* 1987). In contrast to the other neuropeptides studied herein, in previous studies SP always had an inhibitory effect on methacholine-induced sweating in humans (Kumazawa *et al.* 1994; Berg *et al.* 1995). Our functional data confirm this inhibitory effect, which was very modest. We used reasonably low concentrations of SP, which were, however, probably still higher than the physiological concentration of SP in human skin (10^{-9} mol SP dissolved in a volume of 1 mm^3 of skin (Sauerstein *et al.* 2000)), in particular, those doses which suppressed sweating most effectively (10^{-6} and 10^{-7} M). This fact limits our observation and it is well possible that SP has no physiological effect on sweat glands *in vivo*. The mechanism by which SP could suppress sweating is still unclear. The most likely explanation is the oedema, which occludes sweat ducts, in particular if higher concentrations of SP are applied (Leis *et al.* 2003).

Effects on vasodilatation

It has been shown previously that CGRP, VIP and SP increase skin blood flow by vasodilatation (Brain & Williams, 1988; Piedimonte *et al.* 1993; Weidner *et al.* 2000; Bennett *et al.* 2003). Compared with VIP and SP, CGRP is by far the strongest vasodilator among the neuropeptides in human skin (Brain *et al.* 1986). In our study, neuropeptides were not assessed in a way that allows us to draw final conclusions on the vasodilator effects, since we always co-applied ACh. Acetylcholine itself induces a strong vasodilatation by direct effect on endothelial cells and by axon reflex mechanisms (Rukwied & Heyer, 1998; Schlereth *et al.* 2005*a*). We therefore could only look for further enhancement of skin blood flow by the neuropeptides investigated in our study. We found no effect of VIP and SP, but CGRP further enhanced ACh-induced vasodilatation. Thus, we were able to confirm that CGRP is the strongest vasodilator. Interestingly, CGRP mainly amplifies skin blood flow in concentrations which also most effectively increase sweating. One explanation why 10^{-7} and 10^{-9} M CGRP were more effective than 10^{-8} M to induce vasodilatation could be that the degradation route of CGRP differs with different concentrations. As shown for axon reflex vasodilatation, different degradation products of peptidases could have very different vasodilatory potency (Kramer *et al.* 2006). Nevertheless, our data suggest that vasodilatation may be another factor for CGRP-induced hyperhidrosis. Vasodilatation increases skin temperature, and sweating linearly depends on skin temperature (Low & Kennedy, 2002). Furthermore, intact blood flow is the prerequisite for sustained sweating since sweat glands permanently need fluid and blood supply. Stopping the blood flow, e.g. by tourniquet, quickly stops sweating (Ogawa & Low, 1997). Our results suggest that

amplification of sweating could to some extent also be secondary to the increase of skin blood flow. It will be most interesting to dissect direct CGRP fragment effects and indirect vascular effects on peripheral sweating in future studies.

Clinical implications

The major sources of CGRP in human skin are primary afferent neurons, in particular so-called silent nociceptors (Schmelz *et al.* 2000). If these nociceptors were sensitized and then activated, CGRP release would be facilitated, finally leading to regional vasodilatation and skin temperature increase. In complex regional pain syndromes (CRPS) such a mechanism has been postulated for generating symptoms during acute stages (Weber *et al.* 2001). Furthermore, hyperhidrosis regularly occurs in CRPS, and increased peripheral axon reflex sweating has been demonstrated (Birklein *et al.* 1997; Weber *et al.* 2001). Accordingly, increased CGRP concentrations in blood samples from CRPS patients have been found, in particular if hyperhidrosis was present (Birklein *et al.* 2001). These findings, together with the results of the present study, offer an explanation for one enigmatic symptom of acute CRPS – focal hyperhidrosis induced by local CGRP release.

References

Aton SJ, Colwell CS, Harmar AJ, Waschek J & Herzog ED (2005). Vasoactive intestinal polypeptide mediates circadian rhythmicity and synchrony in mammalian clock neurons. *Nat Neurosci* **8**, 476–483.

Bennett AD, Chastain KM & Hulsebosch CE (2000). Alleviation of mechanical and thermal allodynia by $CGRP_{8–37}$ in a rodent model of chronic central pain. *Pain* **86**, 163–175.

Bennett LA, Johnson JM, Stephens DP, Saad AR & Kellogg DL Jr (2003). Evidence for a role for vasoactive intestinal peptide in active vasodilatation in the cutaneous vasculature of humans. *J Physiol* **552**, 223–232.

Berg TJ, Levy DM, Reid G & Abraham RR (1995). The effects of vasoactive intestinal polypeptide and substance P on methacholine-induced sweating and vascular flare in diabetic neuropathy. *Clin Auton Res* **5**, 159–164.

Bickel A, Axelrod FB, Marthol H, Schmelz M & Hilz MJ (2004). Sudomotor function in familial dysautonomia. *J Neurol Neurosurg Psychiatry* **75**, 275–279.

Birklein F, Riedl B, Claus D & Neundorfer B (1998). Pattern of autonomic dysfunction in time course of complex regional pain syndrome. *Clin Auton Res* **8**, 79–85.

Birklein F, Schmelz M, Schifter S & Weber M (2001). The important role of neuropeptides in complex regional pain syndrome. *Neurology* **57**, 2179–2184.

Birklein F, Sittl R, Spitzer A, Claus D, Neundorfer B & Handwerker HO (1997). Sudomotor function in sympathetic reflex dystrophy. *Pain* **69**, 49–54.

Boutsiouki P, Georgiou S & Clough GF (2004). Recovery of nitric oxide from acetylcholine-mediated vasodilatation in human skin in vivo. *Microcirculation* **11**, 249–259.

Brain SD, Tippins JR, Morris HR, MacIntyre I & Williams TJ (1986). Potent vasodilator activity of calcitonin gene-related peptide in human skin. *J Invest Dermatol* **87**, 533–536.

Brain SD & Williams TJ (1988). Substance P regulates the vasodilator activity of calcitonin gene-related peptide. *Nature* **335**, 73–75.

Casey ML, Smith J, Alsabrook G & MacDonald PC (1997). Activation of adenylyl cyclase in human myometrial smooth muscle cells by neuropeptides. *J Clin Endocrinol Metab* **82**, 3087–3092.

Clough GF, Boutsiouki P, Church MK & Michel CC (2002). Effects of blood flow on the in vivo recovery of a small diffusible molecule by microdialysis in human skin. *J Pharmacol Exp Ther* **302**, 681–686.

Dalsgaard CJ, Jernbeck J, Stains W, Kjartansson J, Haegerstrand A, Hokfelt T, Brodin E, Cuello AC & Brown JC (1989). Calcitonin gene-related peptide-like immunoreactivity in nerve fibers in the human skin. Relation to fibers containing substance P-, somatostatin- and vasocactive intestinalpolypeptide-like immunoreactivity. *Histochemistry* **91**, 35–38.

Di Angelantonio S, Giniatullin R, Costa V, Sokolova E & Nistri A (2003). Modulation of neuronal nicotinic receptor function by the neuropeptides CGRP and substance P on autonomic nerve cells. *Br J Pharmacol* **139**, 1061–1073.

Ebner K, Rupniak NM, Saria A & Singewald N (2004). Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats. *Proc Natl Acad Sci U S A* **101**, 4280–4285.

Giniatullin R, Di Angelantonio S, Marchetti C, Sokolova E, Khiroug L & Nistri A (1999). Calcitonin gene-related peptide rapidly downregulates nicotinic receptor function and slowly raises intracellular Ca^{2+} in rat chromaffin cells in vitro. *J Neurosci* **19**, 2945–2953.

Goodnough JE, O'Dorisio TM, Friedman CI & Kim MH (1979). Vasoactive intestinal polypeptide in tissues of the human female reproductive tract. *Am J Obstet Gynecol* **134**, 579–580.

Hagner S, Haberberger RV, Overkamp D, Hoffmann R, Voigt KH & McGregor GP (2002). Expression and distribution of calcitonin receptor-like receptor in human hairy skin. *Peptides* **23**, 109–116.

Hartschuh W, Reinecke M, Weihe E & Yanaihara N (1984). VIP-immunoreactivity in the skin of various mammals: immunohistochemical, radioimmunological and experimental evidence for a dual localization in cutaneous nerves and merkel cells. *Peptides* **5**, 239–245.

Heinz-Erian P, Paul S & Said SI (1986). Receptors for vasoactive intestinal peptide on isolated human sweat glands. *Peptides* **7** (Suppl. 1), 151–154.

Henning RJ & Sawmiller DR (2001). Vasoactive intestinal peptide: cardiovascular effects. *Cardiovasc Res* **49**, 27–37.

Hilz MJ, Axelrod FB, Bickel A, Stemper B, Brys M, Wendelschafer-Crabb G & Kennedy WR (2004). Assessing function and pathology in familial dysautonomia: assessment of temperature perception, sweating and cutaneous innervation. *Brain* **127**, 2090–2098.

Ishida-Yamamoto A & Tohyama M (1990). Calcitonin gene-related peptide-like immunoreactive cells in the secretory portions of rat sweat glands. *Cell Tissue Res* **260**, 621–623.

Kennedy WR, Wendelschafer-Crabb G & Brelje TC (1994). Innervation and vasculature of human sweat glands: an immunohistochemistry-laser scanning confocal fluorescence microscopy study. *J Neurosci* **14**, 6825–6833.

Kerdelhue B, Lenoir V, Kolm P, Seltman HJ, Jones HW Jr & Jones GS (1997). ACTH, beta-endorphin, substance P, and corticotrophin releasing hormone in plasma and follicular fluid in hormonally stimulated menstrual cycles for in-vitro fertilization in the human. *Hum Reprod* **12**, 231–235.

Kramer HH, Schmidt K, Leis S, Schmelz M, Sommer C & Birklein F (2005). Inhibition of neutral endopeptidase (NEP) facilitates neurogenic inflammation. *Exp Neurol* **195**, 179–184.

Kramer HH, Schmidt K, Leis S, Schmelz M, Sommer C & Birklein F (2006). Angiotensin converting enzyme has an inhibitory role in CGRP metabolism in human skin. *Peptides* **27**, 917–920.

Kruger L, Silverman JD, Mantyh PW, Sternini C & Brecha NC (1989). Peripheral patterns of calcitonin-gene-related peptide general somatic sensory innervation: cutaneous and deep terminations. *J Comp Neurol* **280**, 291–302.

Kumazawa K, Sobue G, Mitsuma T & Ogawa T (1994). Modulatory effects of calcitonin gene-related peptide and substance P on human cholinergic sweat secretion. *Clin Auton Res* **4**, 319–322.

Kumazawa K, Sobue G, Mitsuma T, Sugenoya J & Ogawa T (1997). Impairment of calcitonin gene-related peptide-induced potentiation of cholinergic sweat secretion in patients with multiple system atrophy. *Clin Auton Res* **7**, 77–80.

Kummer W, Herbst WM & Heym C (1990). Vasoactive intestinal polypeptide receptor-like immunoreactivity in human sweat glands. *Neurosci Lett* **110**, 239–243.

LaMotte RH, Lundberg LE & Torebjork HE (1992). Pain, hyperalgesia and activity in nociceptive C units in humans after intradermal injection of capsaicin. *J Physiol* **448**, 749–764.

Landis SC & Fredieu JR (1986). Coexistence of calcitonin gene-related peptide and vasoactive intestinal peptide in cholinergic sympathetic innervation of rat sweat glands. *Brain Res* **377**, 177–181.

Leis S, Weber M, Isselmann A, Schmelz M & Birklein F (2003). Substance-P-induced protein extravasation is bilaterally increased in complex regional pain syndrome. *Exp Neurol* **183**, 197–204.

Lembeck F, Donnerer J & Bartho L (1982). Inhibition of neurogenic vasodilation and plasma extravasation by substance P antagonists, somatostatin and [D-Met2, Pro5]enkephalinamide. *Eur J Pharmacol* **85**, 171–176.

Lindberger M, Schroder HD, Schultzberg M, Kristensson K, Persson A, Ostman J & Link H (1989). Nerve fibre studies in skin biopsies in peripheral neuropathies. I. Immunohistochemical analysis of neuropeptides in diabetes mellitus. *J Neurol Sci* **93**, 289–296.

Lindh B & Hokfelt T (1990). Structural and functional aspects of acetylcholine peptide coexistence in the autonomic nervous system. *Prog Brain Res* **84**, 175–191.

Lindh B, Lundberg JM & Hokfelt T (1989*a*). NPY-, galanin-, VIP/PHI-, CGRP- and substance P-immunoreactive neuronal subpopulations in cat autonomic and sensory ganglia and their projections. *Cell Tissue Res* **256**, 259–273.

Lindh B, Pelto-Huikko M, Schalling M, Lundberg JM & Hokfelt T (1989*b*). Substance P mRNA is present in a population of CGRP-immunoreactive cholinergic postganglionic sympathetic neurons of the cat: evidence from combined in situ hybridization and immunohistochemistry. *Neurosci Lett* **107**, 1–5.

Low PA & Kennedy WR (2002). Cutaneous effectors as indicators of abnormal sympathetic function. In *Autonomic Innervation of the Skin*, ed. Morris JL & Gibbins IL, pp. 165–212. Harwood Academic Publisher, Amsterdam

Low PA, Neumann C, Dyck PJ, Fealey RD & Tuck RR (1983). Evaluation of skin vasomotor reflexes by using laser Doppler velocimetry. *Mayo Clin Proc* **58**, 583–592.

Lundberg JM, Anggard A, Emson P, Fahrenkrug J & Hokfelt T (1981). Vasoactive intestinal polypeptide and cholinergic mechanisms in cat nasal mucosa: studies on choline acetyltransferase and release of vasoactive intestinal polypeptide. *Proc Natl Acad Sci U S A* **78**, 5255–5259.

Lundberg JM, Anggard A, Fahrenkrug J, Hokfelt T & Mutt V (1980). Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: functional significance of coexisting transmitters for vasodilation and secretion. *Proc Natl Acad Sci U S A* **77**, 1651–1655.

Ma W & Bisby MA (1998). Increase of calcitonin gene-related peptide immunoreactivity in the axonal fibers of the gracile nuclei of adult and aged rats after complete and partial sciatic nerve injuries. *Exp Neurol* **152**, 137–149.

Nistri A & Di Angelantonio S (2002). Enhancement of neuronal nicotinic receptor activity of rat chromaffin cells by a novel class of peptides. *Ann N Y Acad Sci* **971**, 100–107.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP & Shanahan F (2004). The role of substance P in inflammatory disease. *J Cell Physiol* **201**, 167–180.

O'Flynn NM, Helme RD, Watkins DJ & Burcher E (1989). Autoradiographic localization of substance P binding sites in rat footpad skin. *Neurosci Lett* **106**, 43–48.

Ogawa T & Low PA (1997). Autonomic regulation of temperature and sweating. In *Clinical Autonomic Disorders*, ed.Low PA, pp. 83–96. Lippincott-Raven Publishers, Philadelphia.

Ottesen B, Ulrichsen H, Fahrenkrug J, Larsen JJ, Wagner G, Schierup L & Sondergaard F (1982). Vasoactive intestinal polypeptide and the female genital tract: relationship to reproductive phase and delivery. *Am J Obstet Gynecol* **143**, 414–420.

Petersen LJ, Church MK & Skov PS (1997). Histamine is released in the wheal but not the flare following challenge of human skin in vivo: a microdialysis study. *Clin Exp Allergy* **27**, 284–295.

Piedimonte G, Hoffman JI, Husseini WK, Snider RM, Desai MC & Nadel JA (1993). NK1 receptors mediate neurogenic inflammatory increase in blood flow in rat airways. *J Appl Physiol* **74**, 2462–2468.

Rukwied R & Heyer G (1998). Cutaneous reactions and sensations after intracutaneous injection of vasoactive intestinal polypeptide and acetylcholine in atopic eczema patients and healthy controls. *Arch Dermatol Res* **290**, 198–204.

Sato K (1997). Normal and abnormal sweat gland function. In *Clinical Autonomic Disorders*, ed.Low PA, pp. 97–107. Lippincott-Raven Publishers, Philadelphia.

Sato K & Sato F (1987). Effect of VIP on sweat secretion and cAMP accumulation in isolated simian eccrine glands. *Am J Physiol* **253**, R935–R941.

Sauerstein K, Klede M, Hilliges M & Schmelz M (2000). Electrically evoked neuropeptide release and neurogenic inflammation differ between rat and human skin. *J Physiol* **529**, 803–810.

Schlereth T, Birklein F, Haack KA, Schiffmann S, Kilbinger H, Kirkpatrick CJ & Wessler I (2006). In vivo release of non-neuronal acetylcholine from the human skin as measured by dermal microdialysis: effect of botulinum toxin. *Br J Pharmacol* **147**, 183–187.

Schlereth T, Brosda N & Birklein F (2005*a*). Different mechanisms of sudomotor and vasodilator axon reflexes in human skin. *J Neurol* **252**, (suppl. 2), p47, O163.

Schlereth T, Brosda N & Birklein F (2005*b*). Somatotopic arrangement of sudomotor axon reflex sweating in humans. *Auton Neurosci* **123**, 76–81.

Schlereth T, Brosda N & Birklein F (2005*c*). Spreading of sudomotor axon reflexes in human skin. *Neurology* **26**, 1417–1421.

Schmelz M, Luz O, Averbeck B & Bickel A (1997). Plasma extravasation and neuropeptide release in human skin as measured by intradermal microdialysis. *Neurosci Lett* **230**, 117–120.

Schmelz M, Michael K, Weidner C, Schmidt R, Torebjork HE & Handwerker HO (2000). Which nerve fibers mediate the axon reflex flare in human skin? *Neuroreport* **11**, 645–648.

Schmelz M & Petersen LJ (2001). Neurogenic inflammation in human and rodent skin. *News Physiol Sci* **16**, 33–37.

Schulze E, Witt M, Fink T, Hofer A & Funk RH (1997). Immunohistochemical detection of human skin nerve fibers. *Acta Histochem* **99**, 301–309.

Shibasaki M & Crandall CG (2001). Effect of local acetylcholinesterase inhibition on sweat rate in humans. *J Appl Physiol* **90**, 757–762.

Stevens LM & Landis SC (1987). Development and properties of the secretory response in rat sweat glands: relationship to the induction of cholinergic function in sweat gland innervation. *Dev Biol* **123**, 179–190.

Tainio H, Vaalasti A & Rechardt L (1987). The distribution of substance P-, CGRP-, galanin- and ANP-like immunoreactive nerves in human sweat glands. *Histochem J* **19**, 375–380.

Tanaka E, Uchiyama S & Nakano S (1990). Effects of calcitonin gene-related peptide and vasoactive intestinal peptide on nicotine-induced sweating in man. *J Auton Nerv Syst* **30**, 265–268.

Togel B, Greve B & Raulin C (2002). Current therapeutic strategies for hyperhidrosis: a review. *Eur J Dermatol* **12**, 219–223.

Vaalasti A, Tainio H & Rechardt L (1985). Vasoactive intestinal polypeptide (VIP) -like immunoreactivity in the nerves of human axillary sweat glands. *J Invest Dermatol* **85**, 246–248.

van Leeuwen JS, Koppeschaar HP, Kop WJ, te Velde ER, Hackeng WH & Haspels AA (1992). Calcitonin gene-related peptide, the menstrual cycle and premenstrual syndrome. *Gynecol Endocrinol* **6**, 199–204.

Weber M, Birklein F, Neundorfer B & Schmelz M (2001). Facilitated neurogenic inflammation in complex regional pain syndrome. *Pain* **91**, 251–257.

Weidner C, Klede M, Rukwied R, Lischetzki G, Neisius U, Skov PS, Petersen LJ & Schmelz M (2000). Acute effects of substance P and calcitonin gene-related peptide in human skin – a microdialysis study. *J Invest Dermatol* **115**, 1015–1020.

Yamashita Y, Ogawa T, Ohnishi N, Imamura R & Sugenoya J (1987). Local effect of vasoactive intestinal polypeptide on human sweat-gland function. *Jpn J Physiol* **37**, 929–936.

Zahn S, Leis S, Schick C, Schmelz M & Birklein F (2003). No alpha-adrenoreceptor induced C-fiber activation in healthy human skin. *J Appl Physiol* **96**, 1380–1384.

Zancanaro C, Merigo F, Crescimanno C, Orlandini S & Osculati A (1999). Immunohistochemical evidence suggests intrinsic regulatory activity of human eccrine sweat glands. *J Anat* **194**, 433–444.

Acknowledgements

This work was supported by the German Research Foundation, DFG Bi 579/1 and Bi 579/4 to F.B. This study contains essential parts of the MD thesis of J.O.D. and B.S., which will be submitted to the Faculty of Medicine, Johannes Gutenberg-University, Germany. We thank S. Turner for his critical review of our manuscript.